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The clinical significance of inflammatory cytokines in primary cell culture in endometrial carcinoma

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Abstract

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Appendix A
Supplementary data
Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.molonc.2012.07.002.

Endometrial cancer is the most common malignancy of the female genital tract, and the incidence and mortality rates from this disease are increasing. Although endometrial carcinoma has been regarded as a tissue-specific disease mediated by female sex steroid pathways, considerable evidence implicates a role for an inflammatory response in the development and propagation of endometrial cancer. We hypothesized that if specific patterns of cytokine expression were found to be predictive of adverse outcome, then selective receptor targeting may be a therapeutic option. This study was therefore undertaken to determine the relationship between cytokine production in primary cell culture and clinical outcome in endometrial adenocarcinoma. Fresh endometrial tissues were fractionated into epithelial and stromal fractions and cultured. After 6–7 days, supernatants were collected and cells enumerated. Batched aliquots were assayed using ELISA kits specific for CSF-1, GMCSF, G-CSF, TNF-α, IL-6, IL-8, and VEGF. Data were compared using ANOVA, Fisher's exact, and log rank tests. Increased epithelial VEGF production was observed more often in tumors with Type 2 variants ($p = 0.039$) and when GPR30 receptor expression was high ($p = 0.038$). Although increased stromal VEGF production was detected more often in grade 3 endometrioid tumors ($p = 0.050$), when EGFR expression was high ($p = 0.003$), and/or when ER/PR expression was low ($p = 0.048$), VEGF production did not correlated with overall survival (OS). Increased epithelial CSF-1 and TNF-α production, respectively, were observed more often in tumors with deep myometrial invasion ($p = 0.014$) and advanced stage ($p = 0.018$). Increased CSF-1 (89.5% vs. 42.9%, $p = 0.032$), TNF-α (88.9% vs. 42.9%, $p = 0.032$, and IL-6 (92.3% vs. 61.5%, $p = 0.052$) also correlated with low OS. In Cox multivariate models, CSF-1 was an independent predictor of low survival when stratified by grade ($p = 0.046$) and histology ($p = 0.050$), and TNF-α, when stratified by histology ($p = 0.037$). In this study, high CSF-1, TNF-α, and IL-6 production rates identified patients at greatest risk for death, and may signify patients likely to benefit from receptor-specific therapy.

Keywords

Endometrial carcinoma; Inflammatory cytokines; CSF-1; VEGF; Macrophages; Tumor microenvironment

1. Introduction

The hallmarks of cancer originally reviewed by Hanahan and Weinberg have been recently extended to include inflammation (Hanahan and Weinberg, 2011). This inclusion is in recognition of growing appreciation of the role of immune cells found in the microenvironment of tumors that in many case promote malignancy (Mantovani et al., 2008). Immune cells in the tumor microenvironment respond to and produce a wide range of cytokines, chemokines and growth factors, such as tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF), colony stimulating factor-1 (CSF-1), interleukins (IL-1, IL-6, IL-8) and vascular endothelial growth factor (VEGF) (Balkwill, 2009; Joyce and Pollard, 2009; Mantovani et al., 2008). In some cases, systemic levels of these cytokines have been shown to correlate with extent of disease. For example, in a wide range of cancers, increased local expression and circulating concentrations of CSF-1, a growth factor that regulates the survival, proliferation, and differentiation of cells of the mononuclear macrophage lineage (Tushinski et al., 1982), are associated with poor prognosis (Kacinski et al., 1990; Scholl et al., 1996; Smith et al., 1995). Moreover, macrophage infiltration, especially at the tumor-stromal interface, has also been shown to correlate with lower survival rates for breast, endometrial, and other solid tumors (Bingle et al., 2002; Bingle et al., 2006; Joyce and Pollard, 2009; Miyazono, 2011; Qian and Pollard, 2010).

In the United States, carcinoma of the endometrium is the most common malignancy affecting the female reproductive tract, and now accounts for 6% of all new cancers in US women (Siegel et al., 2011). Epidemiological, pathological, and molecular/genetic studies support the division of endometrial carcinoma into Type 1 and Type 2 tumors. Type 1 tumors account for 75–80% of cases, are of endometrioid histology, arise within a background of hyperplasia, and are associated with high levels of estrogen (ER), progesterone (PR), and epidermal growth factor receptor (EGFR) expression. Type 2 tumors (uterine papillary serous carcinoma [UPSC], clear cell carcinoma, and mixed variants) invade deeply into the myometrium, lymphatic, and vascular spaces, are less likely to be ER or PR positive, and are more likely to recur. This dualistic model continues to be used for risk stratification, although there is considerable overlap with respect to the genetic aberrations involved (Llaurado et al., 2012).

Estrogen exposure is the major risk factor for endometrial cancer. In the normal endometrium, estrogen and progesterone induce the synthesis of several growth factors such as CSF-1, TNFα and VEGF as part of its cyclical physiology in preparation for pregnancy and in repair and re-building following menstruation (Daiter et al., 1992; Pollard, 1990). This suggests that these tissues synthesize these growth factors to modulate the local environment to enhance pregnancy outcomes and maintain endometrial health. We hypothesize that the oncogenic mutations in endometrial cancer cells result in the selection of similar strategies, often independent of hormonal control, such that as tumors evolve, these growth factors enhance malignant epithelial growth and invasion through autocrine and paracrine pathways.

Our previous work in endometrial carcinoma is consistent with this hypothesis, as it indicated that local expression CSF-1 and its transmembrane Class III tyrosine kinase receptor, CSF-1R (Sherr et al., 1985) were correlated with poor prognostic indicators (Smith et al., 1995). In this present study, we postulated that the differential expression of cytokines/growth factors derived from human endometrial carcinoma cells in primary cell culture would correlate with clinical and pathological predictors of outcome, including survival. To test this hypothesis, in a prospective study of endometrial cancer conducted over 11 years, we compared rates of production of CSF-1, VEGF, IL6, IL-8, TNFα, GM-CSF and G-CSF in primary cell culture by known prognostic indicators and by overall survival.

2. Materials and methods

2.1. Ethics statement

The protocol, titled “CSF-1 and Other Cytokines in Human Endometrial Carcinogenesis” was reviewed and approved by the Institutional Review Board of the University of New Mexico Health Science Center (UNMHSC), Albuquerque, New Mexico in 1997, and subsequently, by the Committee on Clinical Investigations of Albert Einstein College of Medicine, Bronx, NY in 2008; the corresponding approval numbers are HRRC 97–243, and 2008–396–007, respectively.

2.1.1. Study design—Previously untreated patients with endometrial carcinoma consenting for hysterectomy were eligible, provided that 1) they were appropriate surgical candidates and 1) had no coexisting malignancy or were at least 5 years post therapy, without evidence of recurrence and 2) did not have severe anemia (not further defined), where serum donation would likely increase the need for transfusion. The surgical procedure consisted of hysterectomy, removal of both ovaries (BSO) if present, inspection of the abdominal cavity, and removal of all disease deemed resectable. Lymphadenectomy was not required for staging, but usually was performed for cases with grade 2 or higher lesions,

significant (≥30%) myometrial invasion, lymph nodes that were palpably enlarged or enlarged on CT imaging, and when the cervix was involved (Creutzberg et al., 2011; Creutzberg et al., 2001; Keys et al., 2004). Radical hysterectomy was performed for gross cervical disease (Cohn et al., 2007). At surgery, each sample was given a unique identifier link to the endometrial cancer database, which is a compilation of patient-specific clinical and pathological data obtained from review of medical records, pathology reports, tumor board disposition, the New Mexico Tumor Registry, and secondary pathology review. Personnel (NDS) responsible performing the assays had no access to patient data. Although serum and paraffin-embedded tissue was always collected, we anticipated that in at least a third of cases there would be insufficient “left over” tissue suitable for cell culture.

The objectives of this study were 1) to determine if cytokine production rates in primary cell culture were stable over calendar time and passage; 2) if there were differences in production rates obtained from different tissue fractions [benign epithelium (BE), benign stroma (BS), malignant epithelium (TE), and malignant stroma (TS)]; and 3) if there were differences in rates of production obtained from primary cell cultures compared to established cell lines. If so, then primary cell culture might be a feasible model to test the impact of receptor agonist/antagonist activity on the behavior of thesecells. The second aim of this study was to determine if rates of cytokine production correlated with clinical and pathological predictors of adverse outcome, and survival. The New Mexico IRB approved enrollment of 50 cases for cell culture assays, with the expectation that assay results from primary cell culture were likely to be obtained in 20 consenting patients. The data we report were obtained from 50 consenting patients diagnosed between 11/15/1996 and 7/20/2000; the last date of follow-up for survival analyses was 11/5/2010.

2.2. Cell culture methods

In 1979, Satyaswaroop and colleagues developed a system for the isolation and culture of normal human endometrial glandular cells (Satyaswaroop et al., 1979). In that system, Dulbec-co’s minimal essential medium (DMEM) was supplemented with fetal bovine serum and bovine insulin, and normal cells were propagated in intact clusters. We found that substituting UltraCulture™ medium supplemented with Serum Supreme improved the probability of cell attachment, and the use of low concentrations of trypsin (0.05% or less) favored cell survival and attachment at replating (Stephens et al., 1996). Morphological studies and cytospin analyses indicated that the purest populations with the best adhesive properties were obtained at passage 0 (P0). For this reason, P0 with 6–7 days of culture was chosen for supernatant collection and cell harvest.

Immediately following hysterectomy and under aseptic conditions, at least 1 g of tumor was harvested from above the basement membrane and where available, counterpart benign epithelium (10 × 10 mm surface epithelium and 3 mm depth) wedge resection of epithelium and stroma), and tumor from metastatic sites (lymph node or omentum) using methods similar to those previously described for primary renal cell culture (Stephens et al., 1996). Each sample collected was placed in separate transport tubes containing sterile UltraCulture™ (BioWittaker, Wakersville, MD) serum-free medium supplemented with penicillin (100 U/ml) and streptomycin (100 μm/ml) for transport to the laboratory on wet ice. Tissues were washed in Dulbecco’s phosphate-buffered saline/penicillin-streptomycin [DPBS/PS] at 37 °C for 20 min. Rinsed tissues were transferred to 150-mm sterile culture dishes, trimmed of necrosis, diced to 5 mm^3 pieces, then digested in 2.5 mg/ml of collagenase type IV-S/DPBS (activity range 0.5–2 FALGPA units/mg) at 37 °C for up to 2 h with gentle agitation. Cells were washed twice with 50 ml DPBS/PS and pelleted by centrifugation at 800 rpm at 4 °C for 5 min. Washed cells were fractionated using autoclaved stainless steel micropore sieves (Newark Wire Cloth Co., Newark, NJ) of decreasing sizes and collected either as “flow-through” (FT; less than the specified pore

size) or “backwash” (BW; larger than the specified pore size). Large debris and undigested tissues were separated by filtration using a 250 μm sieve. Immediately after filtration, backwash fractions were collected by inverting sieves onto 150-mm culture dishes and flushing with DPBS/PS. Each sample was then plated by sieve size and fraction [250 BW, 106 BW, 38 BW and 38 FT] as described in Figure 1S, Schema. Washed cells were pelleted, enriched with UltraCulture™ medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and 2% (v/v) Serum Supreme [UCSS2], and plated into culture flasks. For all samples, cellular morphology and culture confluency were noted and documented (Figure 2S) using an Olympus CK40 inverted phase-contrast microscope outfitted with an Olympus SC35 35 mm camera and green high-contrast filter (Olympus IF550). Stromal fractions refer to 38 FT fractions, and when obtained from tumor vs. benign specimens they were denoted as TS, and BS, respectively; similarly, epithelial and stromal fractions were labeled as BE and BS. Based on morphology studies, all relatively (>90% pure) backwash fractions were considered epithelial in this analysis.

Because cells did not usually adhere to slides after trypsinization, to further assess the percent of epithelial and stromal cells per fraction, cytospin slide preparations obtained at each step of preparation, including prior to initial plating, at 48 h, 72 h, 96 h, and at final harvest (6–7 days) were prepared and evaluated using immunohistochemistry (Figure 2S). Cells were washed in 10-ml volumes of DPBS, fixed in 95% ethanol for 5 min at room temperature, diluted in 95% ethanol and introduced in 100 μl volumes to cytofunnel chambers pre-loaded in a Cytospin II cytocentrifuge (Thermo Shandon, Pittsburgh, PA). Following centrifugation for 5 min at 1500 rpm, slides were allowed to air-dry, checked for cellular distribution and stored at −20 °C. Slides prepared from the cytospins were incubated with the following antibodies [MAK-6 anti-cytokeratin (Zymed Laboratories, San Francisco, CA), AE1/AE3 anti-cytokeratin (Ventana Medical Systems, Tucscon, AZ), anti-CD10 (clone 56C6; Novocastra Laboratories, Ltd. Newcastle upon Tyne, UK) and PG-M1 anti-CD-68 (Dako Corporation, Carpinteria, CA)] in an automated immunostainer (model NexES, Ventana Medical Systems), using dilutions of 1:4, 1:80, and 1:50, respectively. Control tissues included normal skin, kidney, liver or appendix (MAK-6; AE1/AE3), normal thymus, tonsil and appendix (CD10) and lung granuloma (CD68). Grading and interpretation of immunostaining was performed by a senior pathologist (NEJ, Tricore Reference Laboratories, Albuquerque, NM) using previously published methods (Smith et al., 2007).

2.3. ELISA analyses

Three separate ELISA assays were conducted (without free-thaw of any sample), using negative controls (culture media containing FBS and SS) and positive control samples (previously evaluated kidney cells (Stephens et al., 1996), and endometrial cancer cell lines. Sorting was performed with the technicians involved blinded to the identity of cases and controls. Cytokine content was measured in duplicate using quantitative sandwich enzyme immunoassay kits from R&D Systems (Minneapolis, MN) which employ monoclonal capture and polyclonal conjugate antibodies with hydrogen peroxide-tetramethylbenzidine (CSF-1, GM-CSF, IL-6, IL-8, VEGF) or NADPH/INT-violet (TNF-α) detection systems. Conditioned media were analyzed in duplicate wells, undiluted and at dilutions of 1:2, 1:5, and 1:10, 1:17.5, or 1:100. Whenever possible, the production rates we report are those obtained using undiluted samples, or the lowest dilutions within scale of the assay. Optical densities were measured using a Bio-Tek microplate reader programmed for automatic wavelength correction, and were calculated based upon standard curves generated with known amounts of recombinant human cytokine; only values falling within the linear range of the standard curve were used in calculating titers. Assay sensitivities were, respectively: CSF-1, <9 pg/ml; GM-CSF, <2.8 pg/ml; G-CSF <1.71 pg/ml; IL-6, <0.70 pg/ml; TNF-α,

<0.18 pg/ml; IL-8, <3.5 pg/ml, and VEGF, <9 pg/ml. The inter-assay variability (3 repeats) ranged from 2.01 to 7.91 (Whitcomb et al., 2010). Multiple repeats of control samples within the same ELISA to calculate intra-assay variability were not performed.

2.4. Statistical analysis and clinical data

Data were analyzed by SAS (SAS Institute Inc, SAS/STAT User's Guide version 9.1, Cary NC SAS Institute Inc., 2003). Production rates [picograms/10^5 cells/72 h] were compared by cytokine and tissue compartment. Because no "normal" appearing endometrial tissue could be obtained in cases where the endometrial lining was replaced by tumor, benign tissues sufficient to establish cultures were provided in only 12/26 (46.2%); therefore, only the tumor fractions were used for clinical comparisons.

Since production rates varied by several orders of magnitude, data were log transformed for the analyses. Statistical methods for parametric data included ANOVA, repeated measures ANOVA, and paired *t* tests. For each cytokine by fraction, the median production rates were determined across multiple samples for each person. Because of possible non-linear effects of cytokine production rates on outcome, the cut score among the first, second, and third quartiles (q1, q2, q3) that best defined mortality (alive vs. dead) was chosen to dichotomize production rate data. If there were no differences, the median (q2) was used (Figure 1S. caption). The differences in cytokine production rates by clinical and epidemiological variables of interest using these cut scores (Figure 1S, caption) were compared using Fisher's exact tests. Relationships between rates of cytokine production by fraction were also evaluated using Pearson correlations. The LIFETEST procedure (SAS 9.2, Cary, NC) was used to calculate survival curves, and differences in survival were compared using Log-rank tests. The impact of cytokine production and clinical/epidemiological factors on survival was also analyzed using Cox's proportional hazards models, and where statistically significant in univariate analyses these were included in multivariate models. Because the number of patients (26) is small and our hypotheses were exploratory, we report data where p values ≤0.15 were observed, acknowledging that our sample size would be able to detect only very large differences. P values ≤0.05 were considered statistically significant.

3. Results

Illustrated in Figure 1, 50 patients with endometrial carcinoma were enrolled in the study. No cell culture data is available in 24 of these, because 1) no tissue sample was provided (10); no supernatants were collected until later passage (2); cells died (4), or culture samples were deemed not pure or overgrown with fibroblasts (4), in which case the supernatants were discarded. There were no significant differences in the two groups by stage ($p = 0.73$), grade (0.24), histology ($p = 0.27$),use of radiation therapy ($p = 0.25$), overall survival ($p = 0.73$), or survival post radiation therapy ($p = 0.63$).

Relevant clinical and pathological characteristics of the 26 patients where cytokine production rates were calculated are summarized in Table 1. By race/ethnicity, 15 (57.7%) were Hispanic, 8 (30.8%) were non-Hispanic white, 2 (7.7%) were Native American, and 1 (3.8%) was an African-American. By type of hysterectomy performed, 23 (88.5%) underwent abdominal hysterectomy, 1 (3.8%) laparoscopic assisted TVH, and 2 (7.7%) radical hysterectomy. Pelvic radiation therapy and brachytherapy was used in 9 cases (34.6%); no patient received chemotherapy.

3.1. Time course of cytokine production/metastatic cell production patterns

Cell fractions were isolated from endometrial tumors and their metastatic sites (when available) as described in materials and methods. Cells were characterized based upon

morphometric and cytospin interpretation of expression of cytokeratin (epithelial) and CD10 (fibroblastic) and CD68 (macrophage) content, and by harvest (day 6–7); each sample used for these analyses were at least 90% pure. Initially, when production rates of CSF-1, GM-CSF, IL-6, TNF-α, and VEGF obtained from supernatants collected at predetermined time points over 96 h (Figure 2) were evaluated, rates of production compared by the different time points were not significant (all $p > 0.16$). Production rates were higher in cells derived from primary tumors than from metastatic (met) sites for CSF-1 ($p = 0.005$), GM-CSF ($p < 0.001$), IL-6 ($p < 0.001$), and VEGF ($p < 0.001$). There were also differences in rates of production in the primary cell culture tumor fractions (TE vs. TS) for IL-6 ($p = 0.009$) and VEGF ($p = 0.008$) and by cell count ($p = 0.002$). Rates of cytokine production from KLE and RL-95-2 cell lines were not significantly different (DNS), but of note, were consistently significantly lower than rates obtained using primary cell cultures for all cytokines tested [CSF-1, 343.5 vs. 5220.8, $p = 0.005$; TNF-α, 2.04 vs. 692, $p = 0.004$; IL6, 14.0 vs. 52,369.4, $p = 0.001$; and VEGF, 84.7 vs. 1,713, $p = 0.01$]. Because cell counts and production rates in some cases began to decrease after 72 h, this time point was chosen for harvest for the remaining primary cell culture studies.

Supplemental Table 1 depicts the 72-hour mean (± standard error [SE]) rates of production for all primary culture fractions analyzed. The highest production rates were for IL-8, which were significantly higher than rates for IL6, VEGF and CSF-1, which were significantly higher production rates for G-CSF and GM-CSF, which were also significantly higher than production rates for TNF-α (all $p < 0.001$). There were also significant differences in rates of production between benign fractions [BE vs. BS] for G-CSF ($p = 0.030$) and VEGF ($p = 0.030$) and tumor fractions [TE vs. TS] for IL-6 ($p = 0.039$), and G-CSF (0.002), and by cell count ($p < 0.001$). As noted, counterpart benign tissues (22 samples) were available for only 12 patients.

3.2. Cytokine production rates by epidemiological clinical parameters

Cytokine production rates were compared by age at diagnosis, and co-morbidities associated with endometrial cancer such as hypertension and diabetes (Table 2). Stromal production of both CSF-1 and VEGF (0 vs. 69.2%, $p = 0.029$) were elevated more often in samples from women 50 years of age or older. Increased stromal production of GM-CSF (p = 0.019) and G-CSF ($p = 0.050$) were observed more often in samples obtained from hypertensive women, and increased stromal GM-CSF, when significant cardiovascular disease (CAD) was present ($p = 0.029$). Epithelial cell production rates of G-CSF and VEGF were reduced in samples collected from obese (defined as >40% ideal body weight, $p = 0.023$). In samples obtained from women who self-reported previous postmenopausal estrogen exposure compared no prior use, elevated production rates of stromal TNF-α ($p = 0.033$) vs. epithelial VEGF ($p = 0.023$) were more frequently observed.

3.3. Cytokine production rates by pathological parameters

Cytokine production rates were compared by clinical and pathological predictors of adverse clinical outcome including International Federation of Gynecology and Obstetrics (FIGO) stage using 2009 criteria, tumor grade, and histological subtype (Table 3). By stage (I/II vs. III/IV) elevated production rates of epithelial cell TNF-α were observed more frequently in samples from women with advanced disease ($p = 0.018$). By histologic subtype (Type 2 vs. 1) increased epithelial cell production of VEGF ($p = 0.039$) and to a lesser extent CSF-1 ($p = 0.110$), and reduced production of G-CSF ($p = 0.070$) were more common. Confining the analysis to endometrioid (Type 1) histology and then stratified by grade (1 + 2 vs. 3), increased production of G-CSF (epithelial cell $p = 0.023$, stromal cell $p = 0.110$) was more frequent in low grade, and VEGF (stromal $p = 0.05$), in high grade tumor fractions.

Increased CSF-1 production rates (TE) were more frequently observed from tumor with deep myometrial invasion (<50% vs. ≥50%, $p = 0.014$).

In the majority of cases, steroid receptor status (ER-α, PR, G-coupled protein receptor-30 [GPR-30], and EGFR) assessed by IHC using median cut scores had previously been determined (Smith et al., 2007). While cytokine expression by either ER or PR status alone was not significant, when used in combination, increased stromal G-CSF production ($p = 0.048$) was more common in samples from tumors with high ER or PR receptor expression, and increased stromal IL-8 ($p = 0.018$) and VEGF ($p = 0.048$) in tumor samples having little or no detectable ER and PR. In our previous study, increased GPR30 expression was more frequently linked to Type 2 tumors, and lower survival rates (Smith et al., 2007). In the current study, epithelial cell GM-CSF ($p = 0.038$), VEGF ($p = 0.038$), and IL-8 ($p = 0.057$) were increased more often in supernatants derived from tumors with increased GPR30 expression.

3.4. Cytokine production rates and overall survival

There were 6/26 deaths from endometrial cancer and three deaths from other causes (pancreatitis, renal failure, and uncontrolled diabetes); excluding these, overall survival rate was 76.9%. In the survival analyses, deaths from other causes were censored events. The median survival time was 120 months (range 3–154 months), and for those who died from endometrial cancer, 39.3 months; the most recent death from endometrial cancer as of 2/12/2012 was on 2/20/2004. As expected, significant differences in survival rates were observed by FIGO stage (I/II vs. III/IV, 95.0% vs. 16.7%, $p < 0.001$) and tumor grade (1 + 2 vs. 3, 93.3% vs. 54.6%, $p = 0.023$). Survival rates were lower for Type 2 histology (60.0% vs. 81.0%, $p = 0.351$) and in women over 60 years of age (63.6% vs. 86.7%, $p = 0.243$), and although not statistically significant in this study, the differences observed are consistent with other larger series (Hamilton et al., 2006). No differences in survival were seen in women with and without hypertension, diabetes mellitus, obesity (all $p > 0.771$), or by use of adjuvant radiation therapy (No vs. Yes, 14/17, 82.3% vs. 6/9, 66.7%, $p = 0.628$). However, in women with women with CAD, survival rates were lower (90.0% vs. 33.3%, $p = 0.003$).

Depicted in Figure 3, survival rates were lower in patients where tumor cell production rates of epithelial cell CSF-1 (89.5% vs. 42.9%, $p = 0.032$) and TNF-α (88.9% vs. 42.9%, $p = 0.032$) were high, and marginally lower rates were also observed in patients with high epithelial cell IL-6 (92.3% vs. 61.5%, $p = 0.052$) and IL-8 (92.3% vs. 61.5%, $p = 0.078$). Higher survival rates were observed in patients where stromal TNF-α (100% vs. 62.5%, $p = 0.055$), and epithelial cell G-CSF (100.0% vs. 75.0%, $p = 0.106$) production rates were high, but these trends were not statistically significant. Irrespective of fraction, VEGF production (low vs. high) did not correlate with survival (epithelial cell VEGF, OS 69.2% vs. 84.6%, $p = 0.224$; stromal cell VEGF, OS 84.6% vs. 80.0%, $p = 0.846$). No significant differencesin overall survival by production rates for the other cytokines evaluated was observed (all $p > 0.299$).

Univariate and multivariate models that adjusted for cytokine expression and clinical parameters are depicted in Table 4. Tumor grade (1 + 2 vs. 3) was an independent predictor of survival ($p = 0.024$), as was stage (I/II vs. III/IV, $p = 0.008$) and CAD ($p = 0.012$), but not histologic subtype ($p = 0.372$) or age at diagnosis ($p = 0.166$). Adjusting for grade, epithelial cell CSF-1 ($p = 0.046$) and IL-6 ($p = 0.054$) production rates were independent predictors of survival, but cytokine production rates were not additive to models adjusting for CAD, stage, or age. Adjusting for tumor type (1 vs. 2) production rates of epithelial cell CSF-1 ($p = 0.05$) and TNF-α (0.037) were significant. Kaplane–Meier curves (Figure 4) illustrate the impact of cytokine production by grade and histology on overall survival,

indicating that survival rates in step-wise fashion were progressively worse, and statistically significant by grade and CSF-1 ($p < 0.001$) and IL-6 ($p = 0.018$). By tumor grade, OS declined from 100% in patients with low-grade tumors where cytokine production rates were low, to 0% and 25%, respectively, in patients with high-grade tumors and high CSF-1/ IL-6 production rates. Similarly, combining histology and CSF-1/ TNF-α, the impact on survival was greater for Type 2 tumors and approached statistical significance (CSF-1, $p = 0.092$; TNF-α, $p = 0.075$).

4. Discussion

In the present study, we used primary cell culture methods to determine which cytokine and growth factors are synthesized locally by endometrial tumor cells, and to determine if production rates correlated with clinical outcome. We demonstrate consistently that high CSF-1 and TNFa, and to a lesser degree IL-6, correlate with low survival and therefore may be markers for directed therapies against these molecules or their receptors.

To our knowledge, this is the only study of cytokine production rates in primary cell cultures derived from fresh endometrial cancer tissues. Using these methods we show that high VEGF production rates by epithelial cells correlated with Type 2 histology and GPR30 overexpression, whereas high stromal production correlated with grade 3 endometrioid tumors, EGFR overexpression, and low or absent ER/PR expression. Nonetheless, in this study, VEGF production rates did not correlate with overall survival rates. In contrast, high tumor epithelial cell CSF-1 levels were linked to extremely poor survival rates, independent of histological subtype or tumor grade. Elevated CSF-1 production rates were associated with three known poor prognostic indicators: high EGFR expression, age at diagnosis, and deep myometrial invasion. In other studies, over-expression of CSF-1 has been associated with poor prognosis in breast, ovarian, endometrial, prostate, and colorectal cancer among others (Kacinski et al., 1990; Mroczko et al., 2007; Smith et al., 1995).

The CSF-1 receptor is expressed on macrophages and in some cases, on malignant tumor cells (Miyazono, 2011; Scholl et al., 1994; Smith et al., 1995), and clinical correlative studies suggest that macrophages are tumor promoting, as their density often is predictive of poor outcome, although with exceptions (Qian and Pollard, 2010). Macrophages secrete potent proinflammatory cytokines such as TNF-α, IL-6, and IL-1b1, which are known to activate numerous transcription factors that regulate the expression of genes involved in immune responses, anti-apoptosis, angiogenesis, and metastasis (Wang et al., 2011). The transcriptomes from tumor-associated macrophages (TAMs) derived from mouse models of breast cancer, compared with human breast cancer databases, suggests that macrophage transcripts can be predictive of poor prognosis and reduced survival (Ojalvo et al., 2009). Similarly, West and colleagues identified a translocation involving CSF-1 that resulted in the recruitment of a predominance of CSF-1R-expressing macrophages, and postulated that this same CSF-1 gene expression signature could be used as a surrogate for macrophage response to CSF-1 in other tumors (West et al., 2006). In breast cancer, this CSF-1 response signature correlated with features linked to poorer outcomes (high grade, decreased estrogen and progesterone expression, and increased TP53 mutations (Hercus et al., 2009).

Experimental models provide additional support to the clinical observations previously discussed. In the Polyoma Middle T oncoprotein (PyMT) mouse model of breast cancer, genetic ablation of the macrophage-derived CSF-1 using the $Csf1^{op}$ null allele resulted in reduced macrophage density coinciding with a slower rate of tumor progression with fewer metastases (Lin et al., 2001). In breast cancer, there is an obligatory paracrine loop between epithelial cells and macrophages that is necessary for tumor cell migration and intravasation that is mediated by CSF-1 and EGF synthesized by tumor cells and macrophages

respectively (Condeelis and Pollard, 2006; Wyckoff et al., 2007) This is consistent with the present study, where epithelial CSF-1 and EGFR expression correlate with poor survival. In endometrial, prostate, breast and ovarian carcinoma, CSF-1R is expressed on the epithelial cells as well, and is also predictive of poor prognosis (Kluger et al., 2004; Llaurado et al., 2012; Smith et al., 1995). Epithelial CSF-1R expression suggests autocrine actions of CSF-1 that have been shown to enhance tumor cell invasiveness in breast cancer models (Patsialou et al., 2009). Together, these data suggest that both the autocrine and paracrine functions of CSF-1 in tumors where CSF-1 is overexpressed may contribute to the poor survival seen in this patient cohort.

TNF-α, a member of the TNF/TNFR cytokine superfamily, exists as both soluble and membrane-integrated proteins that bind, respectively, to distinct receptors, TNFRI and TNFRII. TNF-α expression is constitutively expressed on the surface epithelium in endometrial hyperplasia and cancer (Garcia et al., 1994). In ovarian cancer tumors and cell lines, local and systemic (sera and/or peritoneal) TNF levels inversely correlate with tumor grade and stage (Balkwill, 2009). In endometrial cancer, increased production of TNFRI and TNFRII are also linked to an increased risk for developing the disease (Ohkawara et al., 2007). In the ovarian cancer model, TNF-α is an important component of a malignant cell-autonomous network of inflammatory cytokines that include IL-6, macrophage inhibitory factor (MIF), and VEGF (Kulbe et al., 2007), and experimentally, TNF-α receptor activation increases ovarian cancer growth, metastatic potential, and resistance to chemotherapy (Balkwill, 2009). In the endometrial cancer prototype for Type 1 disease, Ishikawa cell lines are highly susceptible to TNF cytotoxicity, but then rapidly develop resistance (Marth et al., 1990). In other endometrial cancer cell lines, estrogen has been shown to promote endometrial cancer invasion by stimulating TNF-α expression, which in turn induces endometrial stromal expression of hepatocyte growth factor (Choi et al., 2009). IL-6 activation of epithelial cells in preclinical ovarian cancer models also increases survival and chemoresistance via JAK/STAT signaling, and stimulation of endothelial cell IL-6R enhances ovarian epithelial tumor cell migration and the activation of downstream effectors (Nilsson et al., 2005). Together, these studies and many others indicate that the functionality and inflammatory pathways involved are closely linked, and selective targeting of IL-6, TNF-α, and CSF-1 alone or in combination with chemotherapy are ongoing. For example, CSF-1 blockade in mouse xenograft models has been shown to suppress growth of embryonic tumors, colon carcinoma, and breast cancer, (Hernandez et al., 2009; Yu et al., 2008) and can reverse chemoresistance in human mammary tumors (Coward et al., 2011).

In summary, our data indicate that an abundance of soluble CSF-1, IL-6, and/or TNF-α produced locally by the tumor may identify a subset of patients at greatest risk for treatment failure. We were unable to perform a full multivariate analysis by all significant clinical factors because not all cytokines were tested in all subjects and in all fractions, and our number of cases is small. While these results need to be confirmed in a larger cohort, they are consistent with our mechanistic studies linking CSF-1 mediated cross-talk between tumor cells and TAMS as critical for invasion and metastases. Further studies are needed to determine if these cytokines prospectively identify those patients at greatest risk for recurrence, and in patients with recurrent disease, those who might benefit from receptor-specific therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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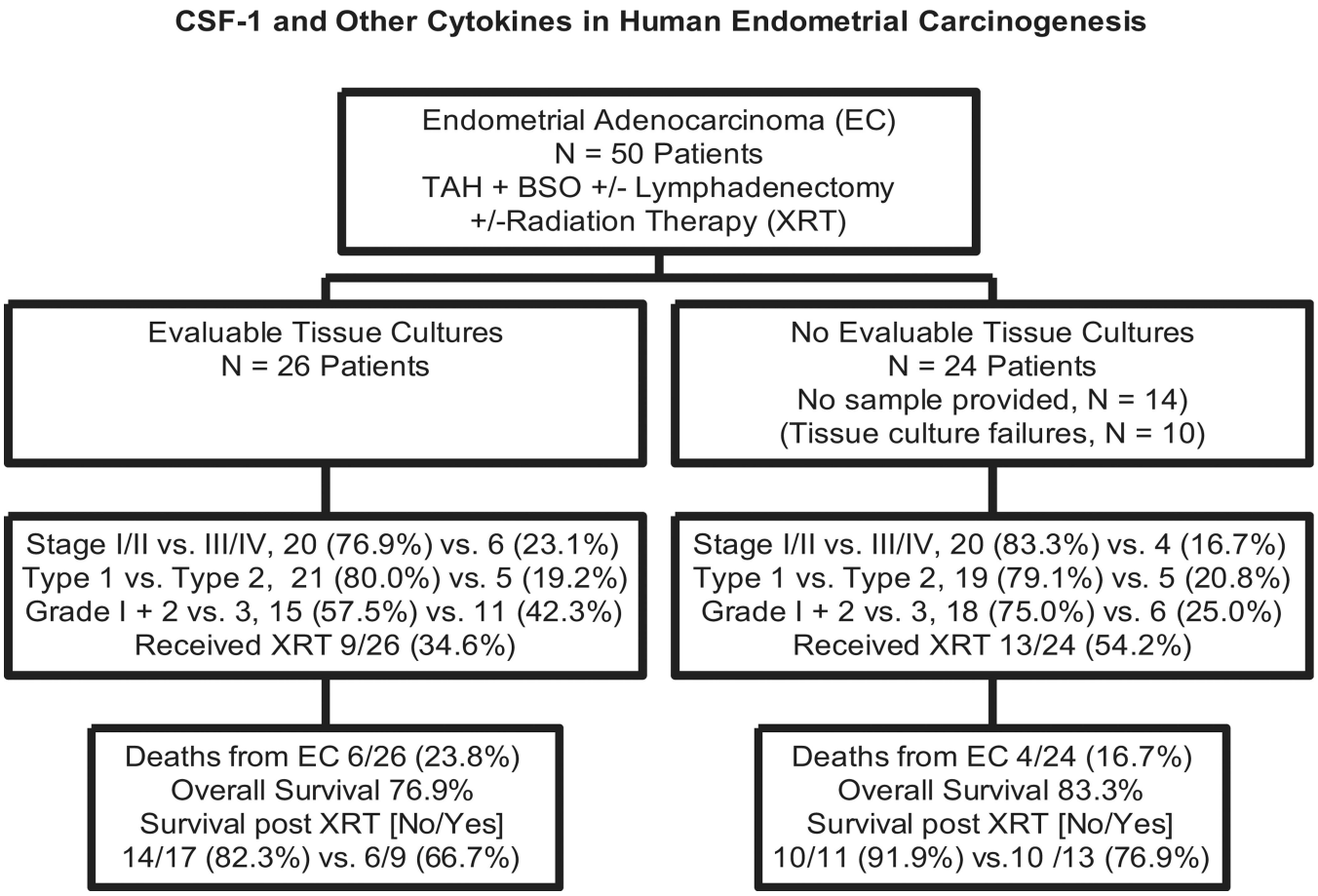

Figure 1.
CSF-1 and other cytokines in human endometrial carcinogenesis. Among the 50 consenting patients, clinical/pathological characteristics, type of therapy given, response to XRT, and overall survival were not significantly different in the group where cell culture was successful ($N = 26$) vs. all others ($N = 24$). Stage (FIGO), defined as I/II vs. III/IV, Grade, defined as [1 + 2] vs. [3], and histology, defined as Type 1 (endometrioid) and Type 2 (uterine papillary serous carcinoma and/or clear cell carcinoma with/without mixed cell features).

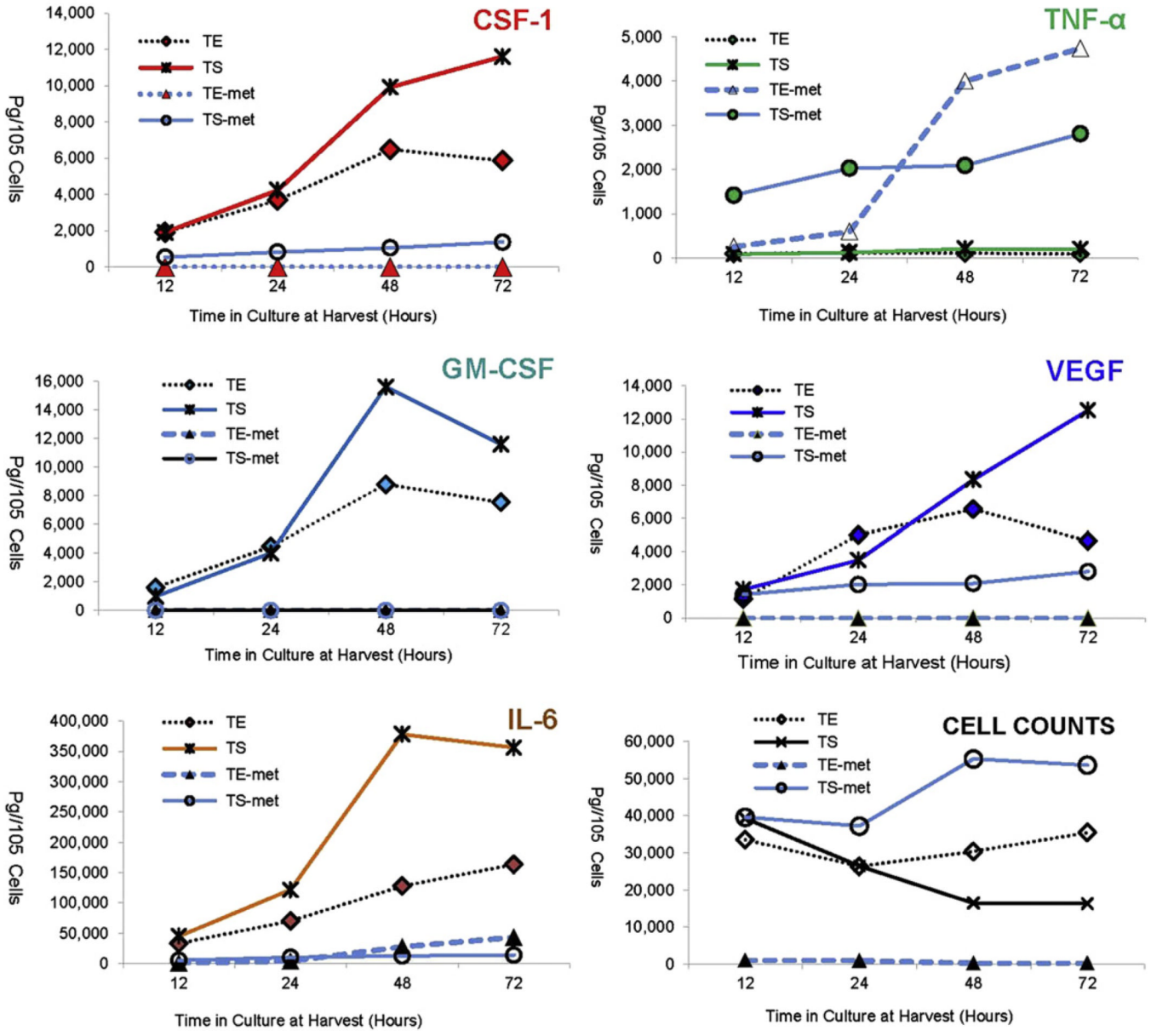

Figure 2.

Time Course of cytokine production *In Vitro*Rates of production (pg/10^5 cells/time interval) of each cytokine by were compared, to determine the impact of the length of time of cells in culture on cell viability and production rates. Fractions evaluated included tumor epithelium (TE), tumor stroma (TS), and metastatic tumor (TE-met/TS-met) collected from omentum or pelvic lymph nodes, segregated by fraction. Cytokines were GM-CSF, granulocyte-macrophage colony stimulating factor, TNF-α, tumor necrosis factor-α, VEGF, vascular endothelial growth factor, CSF-1, colony stimulating factor-1, and IL-6, interleukin-6. Counts depict the number of cells harvested per fraction at each time point. The 72-hour time point was selected for harvest and analyses for subsequent cases.

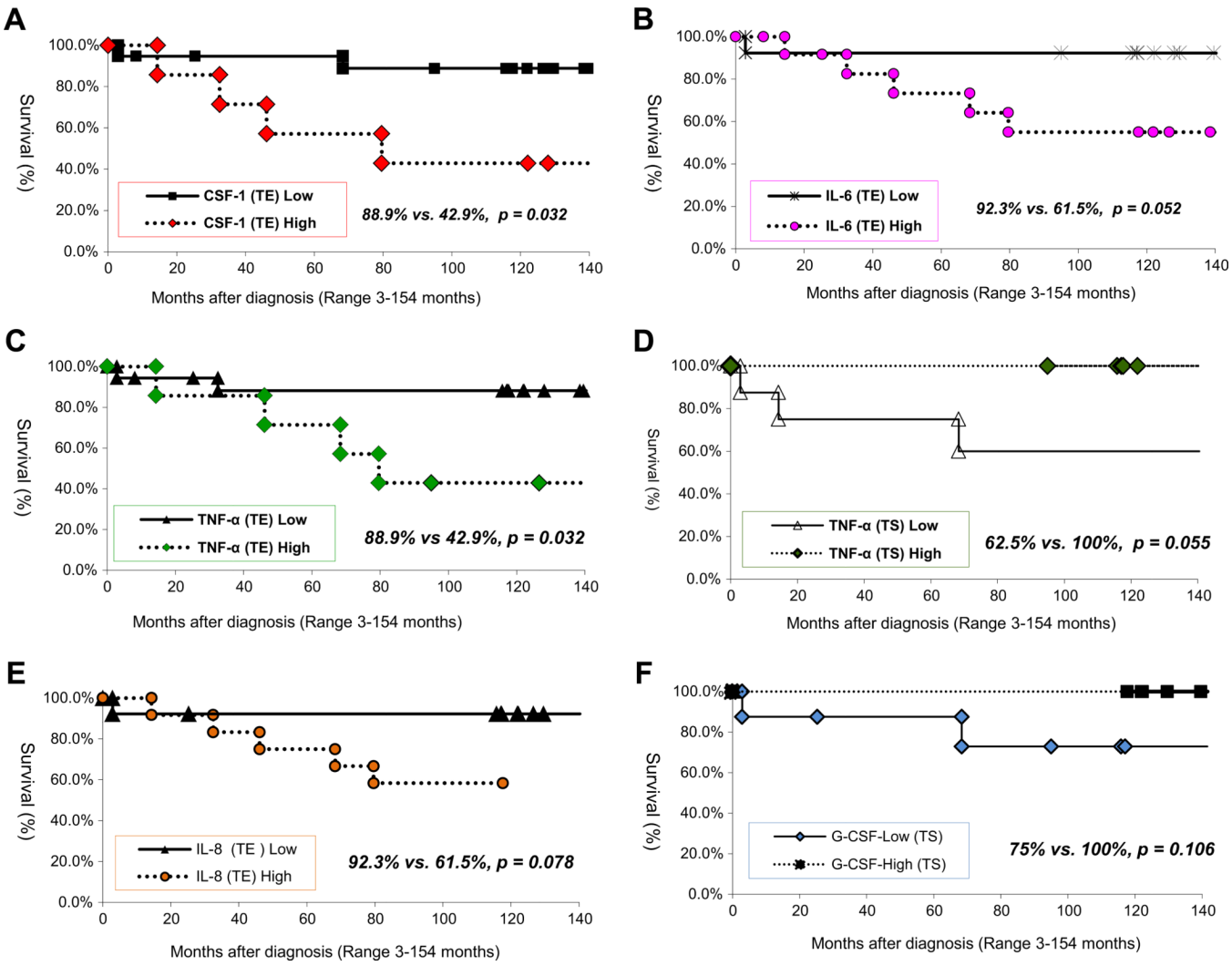

Figure 3.

Overall survival curves by cytokine production rates. Univariate Kaplan–Meier overall survival curves by cytokine and fraction, using median cut scores to dichotomize production rates as high or low, depicting all results with *p* values ≤0.15. A, CSF-1 Production by tumor epithelium (TE); B, IL-6 Production by TE; C, TNF-α Expression by TE; D, TNF-α Expression by TS; E, IL-8 Expression by TE; F, G-CSF Expression by tumor stroma (TS). Cytokines defined in Fig. 1.

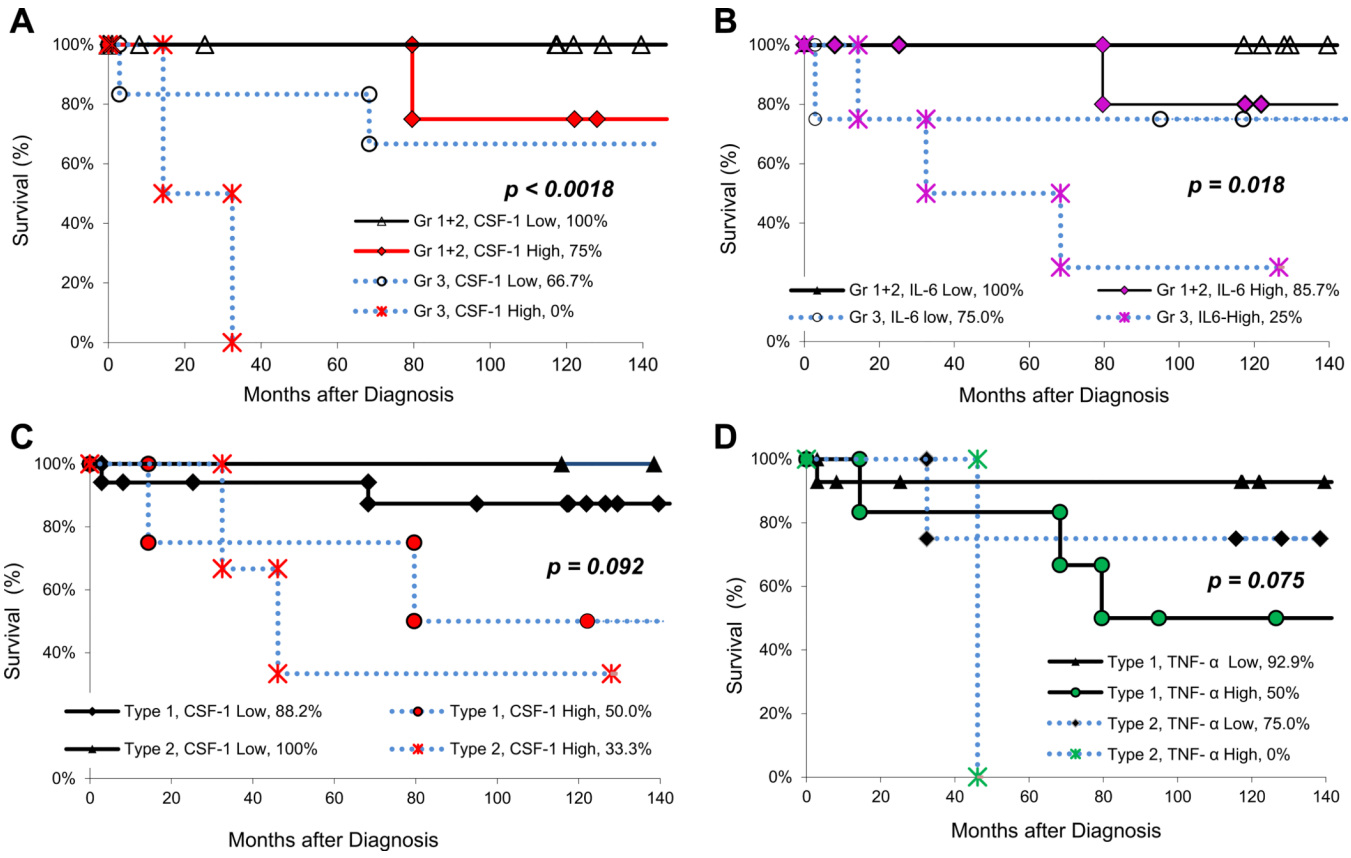

Figure 4.

Multivariate survival by cytokine production rates. Multivariate Kaplane–Meier overall survival curves by cytokine (tumor epithelium), using median cut scores to dichotomize production rates as high or low, depicting all results with *p* values ≤ 0.150. Grade was defined as low-[1 + 2] and high-risk [3], and histology as Type 1 (endometrioid) and Type 2 (uterine papillary serous carcinoma). A, CSF-1 production by grade; B, IL-6 production by grade; C, CSF-1 production by histology, and D, TNF-α production by histology; CSF-1, colony stimulating factor-1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.

Table 1

Clinical and pathological.

Pathological factors	Number (%)	Factors	Number (%)
FIGO surgical stage		Age at diagnosis	
I/II	20 (76.9%)	<60 years	15 (57.7%)
III/IV	6 (23.1%)	≥60 years	11 (43.2%)
Histology type		Age at diagnosis	
Type 1	21 (80.0%)	<70 years	24 (92.3%)
Type 2	5 (19.2%)	≥70 years	2 (7.7%)
Tumor grade		Obesity	
1+2	15 (57.5%)	No	16 (61.5%)
3	11 (42.3%)	Yes	10 (38.5%)
Endometrioid only	15	Hispanic	
Grade 1 + 2	15 (57.7%)	No	8 (30.8%)
Grade 3	8 (33.3%)	Yes	15 (57.7%)
Uterine tumor volume		Diabetes	
<2 cm	6 (23.1%)	No	19 (73.1%)
≥cm	20 (76.9%)	Yes	7 (26.9%)
Myometrial invasion		CAD	
<50%	18 (69.2%)	No	21 (80.8%)
≥50%	8 (30.8%)	Yes	5 (19.2%)
Cervix involved		Hypertension	
No or glandular only	20 (76.9%)	No	18 (69.2%)
Yes	6 (23.1%)	Yes	8 (30.8%)
LVI		Estrogen use	
No	21 (80.8%)	No	21 (80.8%)
Yes	5 (19.2%)	Yes	5 (19.2%)
Lymph node status		Progesterone use	
Negative	11 (42.3%)	No	18 (69.2%)
Positive	2 (7.7%)	Yes	8 (30.8%)
No biopsies	13 (50.0%)		

Type 1, endometrioid adenocarcinoma; **Type 2**, papillary serous or clear cell carcinoma; **Endometrioid** Only, Grade, restricted to cases with endometrioid features including 2 cases with UPSC histology; **LVI**, lymphovascular space involvement; **Myometrial invasion**, depth of myometrial invasion; **Lymph Node Status**, pelvic and/or paraaortic lymph node status; **Hispani**c, Hispanic vs. non-hispanic white; **CAD**, defined as a recent or remote myocardial infarction, significant aberrations in EKG or echocardiography findings, bypass surgery/stent placement; **Obesity**, defined as ≥40% of ideal body weight (BMI ≥ 40); **Estrogen**, postmenopausal estrogen (± progesterone) use; **Progesterone**, postmenopausal progestin (±).

Table 2

Cytokine expression by epidemiologic factors.A

Epidemiological factors	Cytokine	[a]Cytokine frequency by fact	*P*-value
Age at diagnosis			
<50 vs. ≥50 years old	CSF-1 (TS)	0/5 (0%) vs. 9/13 (69.2%)	***0.029***
	VEGF (TS)	0/5 (0%) vs. 9/13 (69.2%)	***0.029***
<60 vs. ≥60 years old	G-CSF (TE)	14/15 (93.3%) vs. 5/10 (50.0%)	***0.023***
	IL8 (TE)	2/15 (13.3%) vs. 5/10 (40.0%)	0.08
<70 vs. ≥70 years old	TNF-α (TE)	4/22 (18.2%) vs. 2/2 (100.0%)	*0.054*
	IL-6 (TE)	5/23 (21.7%) vs. 2/2 (100.0%)	0.07
Hispanic (No vs. Yes)	IL-6 (TE)	9/16 (56.3%) vs. 1/7 (14.3%)	*0.09*
	VEGF (TS)	8/12 (66.7%) vs. 1/6 (16.7%)	0.13
Hypertension (No vs. Yes)	GM-CSF (TS)	0/9 (0%) vs. 4/7 (57.14%)	***0.019***
	G-CSF (TS)	3/10 (30.0%) vs. 6/7 (85.7%)	***0.050***
	IL-6 (TS)	11/13 (81.3%) vs. 1/3 (33.3%)	0.09
CAD (No vs. Yes)	CSF-1 (TE)	4/20 (20.0%) vs. 3/5 (60.0%)	0.11
	GM-CSF (TE)	4/20 (20.0%) vs. 3/5 (60.0%)	0.11
	TNF-α (TE)	3/19 (15.8%) vs. 3/5 (60.0%)	0.08
	IL8 (TE)	4/20 (20.0%) vs. 3/5 (60.0%)	0.11
	CSF-1 (TS)	12/14 (85.7%) vs. 1/3 (33.3%)	0.12
	GM-CSF (TS)	9/13 (69.2%) vs. 0/4 (0.0%)	***0.029***
	G-CSF (TS)	9/14 (64.3%) vs. 0/3 (0.0%)	*0.08*
	IL-6 (TS)	11/13 (84.6%) vs. 1/3 (33.3%)	0.14
Diabetes (No vs. Yes)	GM-CSF (TE)	3/18 (16.7%) vs. 4/7 (57.1%)	0.07
	IL-8 (TE)	3/18 (16.7%) vs. 4/7 (57.1%)	0.07
	VEGF (TE)	3/18 (16.7%) vs. 4/7 (57.1%)	0.07
	CSF-1 (TS)	2/12 (16.7%) vs. 3/5 (60.0%)	0.12
	VEGF (TS)	2/12 (16.7%) vs. 3/5 (60.0%)	0.12
Obesity (No vs. Yes)	G-CSF (TE)	7/16 (43.8%) vs. 0/10 (0.0%)	***0.023***
	VEGF (TE)	7/17 (43.8%) vs. 0/10 (0.0%)	***0.023***
Estrogen use (No vs. Yes)	VEGF (TE)	18/21 (85.7%) vs. 1/4 (25.0%)	***0.031***
	GM-CSF (TS)	2/13 (15.4%) vs. 2/3 (66.7%)	0.14
	TNF-α (TS)	1/11 (9.1%) vs. 3/4 (75.0%)	***0.033***
Progesterone use (No vs. Yes)	TNF-α (TE)	6/17 (35.3%) vs. 0/7 (0.0%)	0.13
	G-CSF (TS)	1/10 (10.0%) vs. 3/6 (50.0%)	0.12
	TNF-α (TS)	1/10 (10.0%) vs. 3/5 (60.0%)	0.08

Italics and bold signify statistically significant differences.

Hispanic, Hispanic vs. non-hispanic white; **CAD**, defined as a recent or remote myocardial infarction, significant aberrations in EKG or echocardiography findings, bypass surgery/stent placement; **Obesity**, defined as ≥40% of ideal body weight; **Estrogen**, postmenopausal estrogen (± progesterone) use; **Progesterone**, postmenopausal progestin (± estrogen) use. For clarity, where significant results are in bold and *p* values are reported to the third decimal place.

[a]**Cytokine Frequency by Factor**, note that denominators may not sum to 26 because of missing values, especially for tumor stroma.

Table 3

Cytokine expression by clinical/pathological predictors of survival.

Pathological factors	Cytokine	[a]Cytokine frequency by factor	*P* value
FIGO stage (I/II vs. III/IV)	TNF-α (TE)	2/18 (11.1%) vs. 4/6 (66.7%)	**0.018**
Histology type Type 1 vs. Type 2	CSF-1 (TE)	4/20 (20.0%) vs. 3/6 (60.0%)	0.11
	GM-CSF (TE)	14/20 (70.0%) vs. 5/5 (100.0%)	*0.07*
	VEGF (TE)	8/21 (38.1%) vs. 5/5 (100.0%)	***0.039***
Tumor grade 1 + 2 vs. 3	G-CSF (TE)	14/15 (93.3%) vs. 5/10 (50%)	***0.023***
	VEGF (TE)	2/15 (13.3%) vs. 5/10 (50.0%)	*0.075*
	G-CSF (TS)	8/11 (72.7%) vs. 1/6 (16.67%)	***0.050***
	VEGF (TS)	3/11 (27.27%) vs. 6/7 (85.71%)	***0.050***
Endometrioid grade 1 + 2 vs. 3	G-CSF (TE)	14/15 (93.3%) vs. 3/7 (42.9%)	***0.021***
	G-CSF (TS)	8/11 (72.73%) vs. 1/5 (20.0%)	0.11
	VEGF (TS)	3/11 (27.27%) vs. 5/6 (83.3%)	***0.050***
Uterine tumor volume <2 cm vs. ≥cm	G-CSF (TE)	2/5 (40.0%) vs. 17/20 (85.0%)	*0.07*
	VEGF (TE)	3/5 (60.0%) vs. 4/20 (20.0%)	0.11
LVI No vs. Yes	TNF-α (TE)	3/19 (15.8%) vs. 3/5 (60.0%)	*0.08*
	VEGF (TE)	4/20 (20.0%) vs. 3/5 (60.0%)	0.11
	IL6 (TS)	2/13 (15.4%) vs. 2/3 (66.7%)	0.14
Myometrial invasion (<50% vs. ≥50%)	CSF-1 (TE)	2/18 (11.1%) vs. 5/8 (62.5%)	***0.014***
PR or ER (Low vs. High)	G-CSF (TS)	0/4 (0.0%) vs. 4/5 (80.0%)	***0.048***
7 (41.2%) vs. 10 (58.8%)	IL-8 (TE)	2/9 (22.2%) vs. 4/6 (66.7%)	0.14
	IL-6 (TS)	4/5 (80.0%) vs. 0/3 (0.0%)	0.14
PR and ER (Low vs. High)	IL-8 (TS)	5/5 (100.0%) vs. 0/3 (0.0%)	***0.018***
9 (65.3%) vs. 7 (42.7%)	VEGF (TS)	5/6 (83.3%) vs. 0/3 (0.0%)	***0.048***
GPR30 (Low vs. High)	GM-CSF (TE)	1/12 (8.3%) vs. 4/7 (57.1%)	***0.038***
13 (65.0%) vs. 7 (35.0%)	IL-8 (TE)	4/12 (33.3%) vs. 6/7 (85.7%)	*0.06*
	VEGF (TE)	1/12 (8.3%) vs. 4/7 (57.1%)	***0.038***
EGFR (Low vs. High)	CSF-1 (TE)	0/2 (0.0%) vs. 4/4 (100.0%)	*0.07*
12 (46.1%) vs. 14 (53.9%)	TNF-α (TE)	1/11 (9.1%) vs. 6/14 (42.9%)	*0.09*
	CSF-1 (TS)	2/9 (22.2%) vs. 7/9 (77.8%)	*0.06*
	IL-6 (TS)	1/9 (11.1%) vs. 4/8 (50.0%)	0.13
	VEGF (TS)	1/9 (11.1%) vs. 8/9 (88.9%)	***0.003***

Italics and bold signify statistically significant differences.

TE, tumor epithelium; **TS**, tumor stroma; **Type 1**, endometrioid adenocarcinoma; **Type 2**, papillary serous or clear cell carcinoma; **Endometrioid**, restricted to cases with endometrioid features, including 2 UPSC/Clear cell cases with endometrioid features; **LVI**, lymphovascular space involvement; **Myometrial invasion**, depth of myometrial invasion; IHC, immunohistochemistry; **PR or ER** (either receptor is negative or positive); PR and ER, both receptors are either negative or positive; **GPR30**, G-coupled protein receptor 30; **EGFR**, epidermal growth factor receptor. For clarity, where significant results are in bold and *p* values are reported to the third decimal place.

[a]Note that denominators may not sum to 26 because of missing values, especially for tumor stroma.

Table 4

Univariate and multivariate survival by cytokine expression and factor.

Factors in models	Hazard ratio	95% CI	Factor *P* value	[a]Overall *P* value
Age at diagnosis <60 vs. ≥60 years	1.052	0.98, 1.13	0.166	0.166
Tumor grade Grade 1 + 2 vs. 3	2.86	0.98, 8.38	0.056	0.056
Tumor grade	2.78	0.94, 8.23	0.065	*0.024*
CSF-1 (TE)	5.83	1.03, 33.0	*0.046*	
Tumor grade	3.27	1.10, 9.76	0.034	*0.023*
IL-6 (TE)	8.66	0.97, 77.43	*0.054*	
Tumor grade	2.28	0.75, 6.95	0.148	0.076
TNF-α (TE)	3.29	0.56, 19.23	0.187	
Tumor type Type 1 vs. type 2	2.17	0.40, 11.94	0.372	0.372
Tumor type	1.22	0.20, 7.26	0.850	*0.109*
CSF-1 (TE)	5.88	1.00, 34.58	*0.050*	
Tumor type	3.16	0.52, 19.12	0.209	*0.086*
TNF-α (TE)	6.58	1.12, 38.62	*0.037*	
CAD	8.86	1.61, 48.69	*0.012*	*0.012*
CAD	8.65	1.56, 47.92	*0.014*	
IL-6 (TE)	6.22	0.72, 54.05	0.097	
CAD	9.23	1.66, 51.31	*0.011*	*0.012*
IL-8 (TE)	5.84	0.67, 50.60	0.109	
CAD	6.07	1.03, 36.0	*0.047*	*0.016*
CSF-1 (TE)	3.79	0.64, 22.55	0.143	
CAD	6.20	1.07, 35.98	*0.042*	*0.021*
TNFα (TE)	3.48	0.60, 20.19	0.165	
Stage I/II vs. III/IV	24.57	2.83, 213.75	*0.004*	*0.004*
Stage	17.85	1.91, 167.12	*0.012*	*0.008*
CSF-1 (TE)	2.75	0.47, 16.19	0.263	
Stage	19.82	1.86, 211.10	0.012	*0.017*
TNFα (TE)	1.37	0.21, 9.04	0.740	

For each parameter, the univariate model is depicted first, followed by bivariate models; **Type 1**, endometrioid adenocarcinoma; **Type 2**, uterine papillary serous carcinoma or clear cell carcinoma; **CAD**, cardiovascular disease.

[a]Overall *p* value for multivariate model fit (Wald).